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Generation of Cloned Mice by Direct Nuclear Transfer from Natural Killer T Cells

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Summary

Cloning mammals by nuclear transfer (NT) remains inefficient. One fundamental question is whether clones have really been derived from differentiated cells rather than from rare stem cells present in donor-cell samples. To date, cells, such as mature lymphocytes, with genetic differentiation markers have been cloned to generate mice only via a two-step NT involving embryonic stem (ES) cell generation and tetraploid complementation [1–3]. Here, we show that the genome of a unique T-cell population, natural killer T (NKT) cells, can be fully reprogrammed by a single-step NT. The pups and their placentas possessed the rearranged *TCR* loci specific for NKT cells. The NKT-cell-cloned embryos had a high developmental potential in vitro: Most (71%) developed to the morula/blastocyst stage, in marked contrast to embryos from peripheral blood T cells (12%; $p < 1 \times 10^{-25}$). Furthermore, ES cell lines were efficiently established from these NKT-cell blastocysts. These findings clearly indicate a high level of plasticity in the NKT-cell genome. Thus, differentiation of the genome is not always a barrier to NT cloning for either reproductive or therapeutic purposes, so we can now postulate that at least some mammals cloned to date have indeed been derived from differentiated donor cells.

Results and Discussion

NKT-Cell Clones Have High Developmental Potency In Vitro

Previously, we analyzed the efficiency of somatic-cell cloning in mice by using two cell types and six genotypes for donors (2 × 6 factorial analysis of variance [ANOVA]) and found that immature Sertoli cells with the (B6 × 129) F1-mouse-strain genotype gave the best results in terms of birth rates of offspring after embryo transfer (about 10%) [4]. To extend this result, we sought cells that retain high genome reprogrammability among lymphocyte populations from such males. We eventually found that natural killer T lymphocytes (NKT cells) had such potency. NKT cells are small lymphoid cells that play regulatory roles, such as the inhibition of tumor development, protection against the development of autoimmune disease, and maintenance of transplantation tolerance [5], in the immune system. Furthermore, it has recently been demonstrated that NKT cells provide an innate-type immune response to certain glycosphingolipid-bearing microorganisms through recognition by their antigen receptor [6, 7]. We isolated NKT cells by fluorescence-activated cell sorting (FACS) of mononuclear cells from the livers of (B6 × 129) F1 male mice and used these for nuclear transfer (NT). NT was performed by direct injection into enucleated oocytes, as described previously [8]. Peripheral blood (helper) T cells isolated from the same strain of mice were used for NT experiments as controls. After 24 hr in culture, the majority of reconstructed oocytes developed into 2-cell embryos irrespectively of the cells used, as expected from their G0 cell-cycle state (Table 1 and Figure 1A). It is known that G0/G1-stage cell-cycle donors are readily synchronized with oocytes in this cloning technique [8]. Within the next 24 hr, whereas many of the T-cell clones arrested their development at the 2-cell stage, most NKT-cell clones reached the 4-cell stage (Table 1 and Figure 1A). Moreover, 71% of NKT-cell-clone embryos developed into the morulae/blastocyst stage, which was very significantly different from the rate of development of T-cell clones (12%, $p < 1 \times 10^{-25}$) (Table 1 and Figure 1A). This poor development of T-cell clones in vitro is consistent with that reported previously by Hochedlinger et al. [1]. However, the developmental potential of NKT-cell-clone embryos was unexpectedly high. In embryos constructed by NT, normal embryonic gene activation occurs only when the donor genome has been reprogrammed to the zygotic state [9]. For mice, most embryonic gene activation occurs in the early 2-cell stage [10], and, therefore, embryos that fail to activate their zygotic genes do not develop beyond this stage. Thus, the genome of NKT cells appears to be more readily reprogrammed than that of T cells, at least in terms of activation of the genes necessary for preimplantation development.

We further examined the plasticity of the NKT-cell genome by isolating nuclear-transfer ES cell (NTES cell) lines from NKT-cell embryos. When these embryos were harvested under conventional condition for NTES-cell establishment, six lines were successfully isolated. This efficiency (4% per 147 reconstructed embryos) was similar to that reported for clones from cumulus cells (2.3%–6.9%) or adult fibroblasts (1.1%–3.8%) [11] and much higher than the rates reported for clones from lymphocytes (0.2%–0.3%) [1].

The NKT-Cell Genome Supports Full-Term-Embryo Development by Single-Step Nuclear Transfer

When 272 embryos derived from NKT cells were transferred into pseudopregnant females, four (1.5%) developed to term offspring and 13 (4.8%) developed into

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Table 1. Development In Vitro and In Vivo of Embryos Cloned from NKT and T Cells

Cell Type	Time in Culture	No. Cultured	No. (%) Cleaved	No. (%) ≥ 4-Cell	No. (%) M & B*	No. Transferred	No. (%) Implanted	No. (%) Fetuses	No. (%) Placenta-Only
NKT cells									
	48 hr	280	260 (93)	241 (86)		185	112 (61)	3 (1.6)	5 (2.7)
	72 hr	292	274 (94)	241 (83)	207 (71)	87	49 (56)	1 (1.1)	8 (9.2)
T cells									
	48 hr	105	62 (59)	21 (20)		21	3 (14)	0 (0)	0 (0)
	72 hr	232	174 (75)	81 (35)	28 (12)	23	0 (0)	0 (0)	0 (0)

* indicates Morulae and blastocysts. See Figure 1A for statistical analysis.

placenta-only conceptuses (Table 1 and Figure 1A). All these 17 clone placentas showed mild or heavy hyperplasia (0.15–0.46 g) two to four times larger than the genotype-matched-control placentas produced by microinsemination (0.08–0.11 g) (Figure 1B); this is characteristic of the abnormal growth patterns of mouse somatic-cell clones [12, 13]. T-cell-clone embryos were also transferred into recipient females, as described above, but very few implanted and none survived through term (Table 1). The T-cell receptor (TCR) repertoire in NKT cells is highly restricted to the invariant Vα14 receptor, which in the mouse is encoded by the genes *Vα14–Jα281* and *Vβ8* [5]. Thus, it is feasible to trace the NKT donor genome in cloned animals. Southern-blot analysis of these clones with specific probes demonstrated that all clones and their placentas inherited the rearranged *TCRVα14* locus (Figure 1C). The *TCRVβ* locus was also rearranged for both alleles (Figure 1C). Polymerase chain reaction (PCR) amplification and DNA sequencing of the rearranged alleles also confirmed the clonality of the NKT-cell-cloned mice. Cloned mouse #1 inherited the rearranged allele of *Vα14–Jα281* derived from the C57BL/6 strain, whereas that of cloned mouse #2 was from the 129/Sv lineage (Figure 2A). Similarly, the *TCRVβ* sequences of clones #1 and #2 had the in-frame configurations *Vβ8S2-D1-Jβ2S5* and *Vβ8S3-D1-Jβ1S4*, respectively (Figure 2B). We then analyzed peripheral blood cells by FACS for the Vβ phenotypes of the two NKT clones. In both clones, unlike the control donor strain, the TCRVβ cells were all positive for TCRVβ8, clearly demonstrating the occurrence of allelic exclusion [14] in these cloned mice (Figure 2C). Clones #1 and #2 grew into normal-looking adults (Figure 1D). They are currently healthy in appearance at

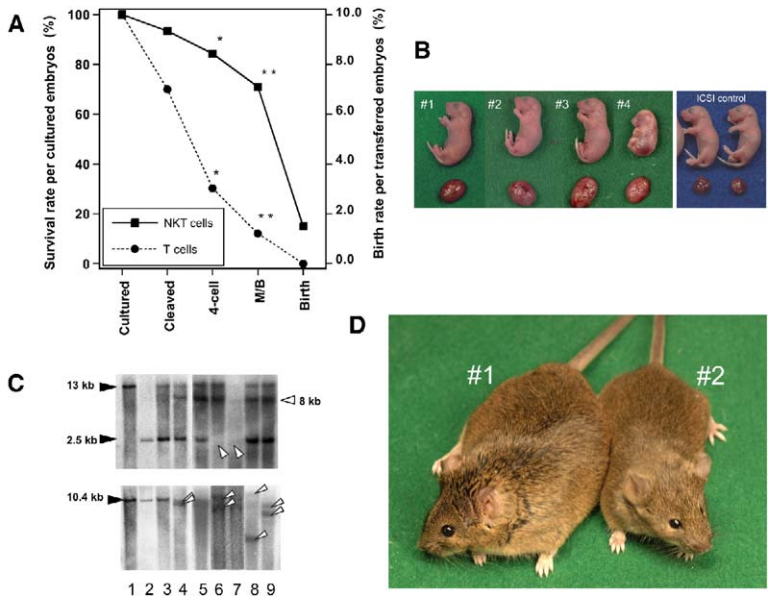

Figure 1. Development and Southern-Blot Analysis of NKT Clones

(A) Survival of embryos reconstructed from nuclei of NKT cells or T cells during in vitro culture. The data from 48-hr- and 72-hr-cultured groups in Table 1 have been combined. * indicates $p < 0.0001$; ** indicates $p < 1 \times 10^{-25}$.

(B) NKT clones and their placentas at birth. Clones #1 and #2 were alive and grew into normal adults (see [D]). Clone #3 was alive at birth but was cannibalized by its foster mother. Clone #4 was stillborn. The clones had placentas that were two to four times heavier than those of the genotype-matched intracytoplasmic sperm injection (ICSI) controls.

(C) Southern-blot analysis of the genomic DNA of NKT-cell-derived cloned mice for the detection of rearrangements of the *TCRVα14* (upper) and *TCRVβ* loci (lower). Black arrowheads indicate the nonrearranged bands, and white arrowheads indicate those that correspond to the rearranged loci. Genomic-DNA samples are from C57BL/6 (B6) mouse tail (lane 1); 129/Sv ES cells (lane 2); donor F1 (B6 × 129/Sv) mouse tail (lane 3); clone #1 tail (lane 4); clone #1 placenta (lane 5); clone #2 tail (lane 6); clone #2 placenta (lane 7); clone #3 placenta (lane 8); and clone #4 placenta (lane 9). At the *Vα14* locus (upper), rearrangement of the B6- and 129-strain alleles is indicated by the appearance of the 8 kb band and the disappearance of the 2.5 kb band, respectively. Clones #1, #3, and #4 carry a rearranged B6-strain allele (lanes 4, 5, 8, and 9), whereas clone #2 carries a rearranged 129-strain allele (lanes 6 and 7; note the absence of the 2.5 kb band; the 8 kb band probably represents an out-of-frame rearrangement of the B6 allele). The 13 kb band for the B6-strain allele persisted after rearrangement because of the presence of a pseudogene. At the *Vβ* locus (lower), both the B6- and 129-strain alleles have been rearranged, as indicated by the appearance of two new bands in clones #1, #2, #3, and #4 (lanes 4 and 5, 6 and 7, 8, and 9, respectively). For clones #1 and #2, rearrangement of the *TCR* loci was also confirmed by sequence analysis (see Figures 2A and 2B).

(D) NKT clones #1 and #2 that grew into adults. They showed a mild obese phenotype which is known to be associated with cumulus-cell clones [17].

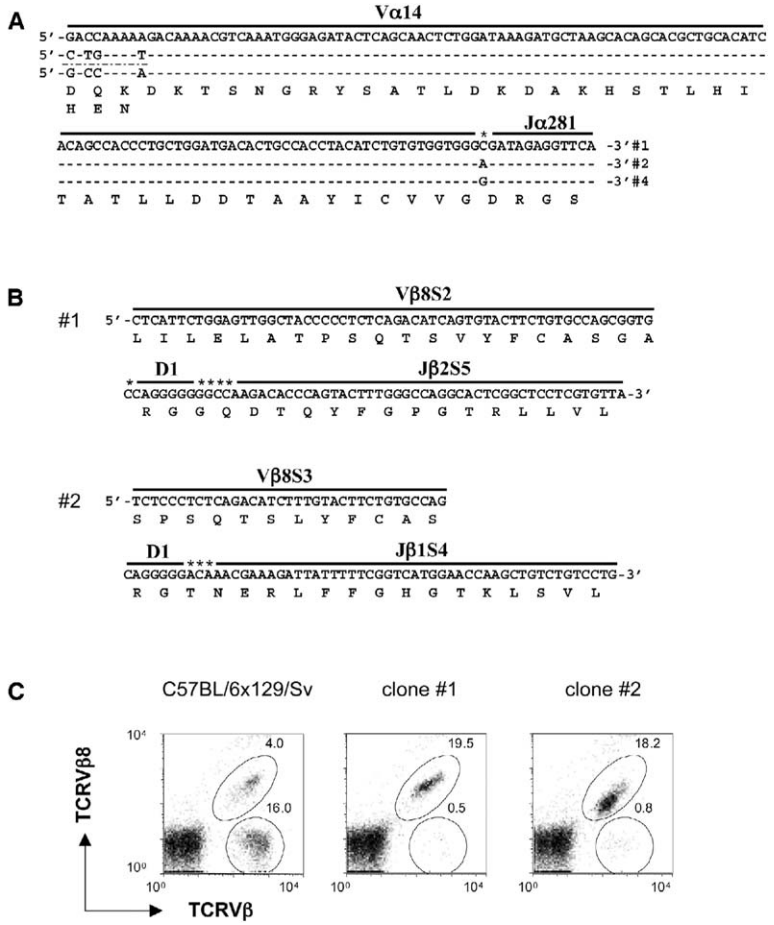

Figure 2. DNA-Sequence Analysis of the *TCRV* Loci and FACS Analysis of Peripheral Lymphocytes in NKT Clones

(A) The *TCRVα14* DNA sequences. The dot-dash line indicates polymorphism between the alleles of the C57BL/6 (B6) and 129 mouse strains. Clones #1 and #4 carry the rearranged B6-strain allele, whereas clone #2 carries the rearranged 129-strain allele. The asterisk indicates the *N*-position nucleotide that connects *Vα14* and *Jα281*. The derived DNA sequences show in-frame rearrangements. The bottom line indicates the amino acid sequence.

(B) The *TCRVβ* DNA sequences. That of clone #1 consists of the in-frame *Vβ8S2-D1-Jβ2S5* sequence, whereas that of clone #2 consists of the in-frame *Vβ8S3-D1-Jβ1S4* sequence.

(C) FACS analysis of peripheral lymphocytes in cloned mice. Expression of *Vβ8* on *TCRVβ*-positive T cells in the peripheral blood from the control (B6 × 129), clone #1, and clone #2 mice. Numbers in circles represent the percentage of gated lymphocytes. Disappearance of non-*Vβ8*-expressing T cells from the *TCRVβ*-carrying population in the two clones demonstrates exclusive expression (allelic exclusion) of the *Vβ8* allele at the *TCRVβ* locus.

the ages of 15 and 11 months, respectively, although they are relatively heavier than normal mice, as reported for mice cloned from cumulus cells [15] (Figure 1D). They proved to be fertile, producing litters of offspring that have normal numbers (nine to 17 pups per litter) and in which the rearranged alleles have been inherited through the germline. These F1 progeny are also fertile and have given birth to F2 mice, some of which are homozygous for the rearranged alleles, as expected (data not shown).

NKT-Cell Cloning as a Model for Nuclear-Transfer Studies

It has previously been reported that the nuclei of peripheral lymphocytes and olfactory sensory neurons, which both express stable markers of differentiated cells, can be cloned to generate mice [1–3]. These findings are very clear demonstrations of the reprogrammability of the differentiated nuclei. However, unlike most other clones, these were generated by a two-step NT, a technique involving ES-cell generation and tetraploid complementation to produce a viable placenta [1–3]. In this, the ES-cell stage allows for extra reprogramming time, and tetraploid-cell lines may contribute to most extra-embryonic tissues, which are commonly the more adversely affected components in cloned animals [16]. Furthermore, tetraploid cells rarely contribute to any part of the embryo proper through term [17]. Eggan et al. also attempted to produce clones directly from neuronal nuclei without tetraploid complementation, but again ES-cell intervention was necessary [2]. These facts leave open the possibility that the "successful" clones may have been produced by the accidental use of undifferentiated stem cells [18]. In this paper, we report the birth of offspring after single-step direct NT from NKT cells. The transferred genomes generated embryonic as well as extra-embryonic tissues, which play essential complementary roles in mammalian development. This provides the first direct evidence that fully differentiated cell nuclei carrying and expressing specific genetic markers can be reprogrammed within the oocyte cytoplasm to support full-term-embryo development. Thus, we can postulate that at least some of the mammals cloned to date are probably derived from differentiated donor cells rather than from stray stem cells. We observed a very high developmental ability of NKT-cell embryos in vitro (71% of the embryos reached the morula/blastocyst stage) and efficient generation of NKT-ES cells (4% per reconstructed embryos). Thus, certain fully differentiated cells may be a good source of donors for reproductive cloning in animals as well as therapeutic cloning in humans.

In mouse NT experiments, cloned offspring are most efficiently obtained when ES cells are used for donors

[19]. By contrast, cloning from T cells and B cells is extremely inefficient, and cloning fetal neural cells [20] is intermediate. Immature Sertoli cells from newborn males were reported to be better donor cells than cumulus cells, the germline support cells in female adults [6]. Taken together, these ideas show that it is conceivable that the efficiency (birth rate) of cloning depends on the state of differentiation of donor cells [21]. However, we demonstrate here that NKT cells with specific differentiated markers are suitable donors for the generation of cloned offspring and NTES-cell lines. Furthermore, although tissue-specific stem cells (e.g., hematopoietic cells and neural stem cells) are expected to be effective donor cells because of their innate differential plasticity [21], so far there has been no report of successful cloning by NT from these cells. Thus, we speculate that the capacity of the genome to be reprogrammed by NT is biologically distinct from the degree of genomic plasticity on the basis of its differentiation status (or its "stemness," in reverse). What determines this potential? Both NKT cells and T cells belong to the same hematopoietic-cell lineage and undergo similar DNA rearrangements to express TCR, but attempts to clone from these lineages show very different efficiency. Examination of the relationship between chromatin structure and the efficiency of cloning from different hematopoietic cells, including NKT cells, might help solve this question.

Experimental Procedures

Preparation of Donor Cells

Male (C57BL/6 × 129/Sv) F1 mice aged 2 to 5 months were used for the preparation of NKT cells and helper/inducer T cells. For the collection of NKT cells, we isolated dispersed liver mononuclear cells by using Percoll density gradients (Amersham Biosciences, Piscataway, New Jersey), and we sorted NKT cells via a Moflo flow cytometer (Dako Cytomation, Carpinteria, California) with phycoerythrin (PE)-α-galactosylceramide-loaded CD1-D tetramer (prepared in-house) and a fluorescein isothiocyanate (FITC)-labeled anti-TCRVβ (H57) monoclonal antibody (PharMingen, San Diego, California). The NKT cells were sorted twice and were more than 99% pure, as assessed by flow cytometry. For the collection of T cells, we isolated mononuclear cells from peripheral blood and stained them with PE-conjugated anti-CD3 and allophycocyanin (APC)-conjugated anti-CD4 monoclonal antibodies (eBioscience, San Diego, California). $CD3^+CD4^+$ helper/inducer T cells were sorted with a triple-laser flow cytometer, model FACS Vantage SE (BD Biosciences, San Jose, California). The purity of sorted cells was determined as more than 98% by flow cytometry. In our preliminary study, we confirmed 97%–99% viability after sorting for both NKT-cell and T-cell populations by propidium-iodide staining.

Nuclear Transfer

Nuclear transfer was performed as described previously, with minor modifications [4, 8]. Mature oocytes were collected from superovulated B6D2F1 females and enucleated and injected with NKT-cell nuclei via a Piezo-driven micromanipulator (Primetech Corporation, Ibaraki, Japan). The reconstructed oocytes were cultured in KSOM medium [22], and 4-cell embryos (48 hr in culture) or morulae/blastocysts (72 hr in culture) were transferred into the oviducts of ICR-strain pseudopregnant females at 0.5 days post coitus (dpc) with vasectomized males. At 19.5 dpc, the recipient females were killed and their uteri were examined for live or dead fetuses. Live fetuses were then reared by lactating ICR-strain foster mothers. Genotype-matched controls were produced by intracytoplasmic sperm injection as previously reported [23].

Southern-Blot Analysis

Genomic DNA (4 μg from the tail or 20 μg from the placenta) was digested with EcoRI (*TCRVα14* probe) or BamHI (*TCRVβ* probe). The bands were visualized with radiolabeled TCR *Vα14* and *Vβ* probes, which were derived from genomic regions 66569–67106 (AE008684) and 163526–164357 (AE000665.1), respectively.

Sequence Analysis

The *Vα14–Jα281* fragment was amplified with AmpliTaq-Gold (Roche, Nutley, New Jersey) in a PCR reaction mixture that contained RNase-treated genomic DNA and the primers 5′-GACC CAAGTGGAGCAGAGTC-3′ and 5′-AGGTATGACAATCAGCTGAG TCC-3′. The PCR products were purified with a PCR Purification Kit (Qiagen, Tokyo, Japan) and sequenced with a model 3100 Genetic Analyzer (Applied Biosystems/Hitachi, Tokyo, Japan). For *TCRVβ* sequencing, we used RNase-treated genomic DNA as the template in the PCR reaction to detect in-frame *Vβ-D-J* fragments. In brief, we used combinations of primers that corresponded to a given *TCRVβ* region and that encompassed either the 5′ end of the *TCRVβC1* gene or the 3′ end of the *J β2S7* gene. The PCR products were sequenced as described above.

Analysis of Peripheral Blood T cells

Lymphocytes were stained with allophycocyanin-labeled anti-TCRVβ (H57–597, PharMingen) and fluorescein-isothiocyanate-labeled anti-TCRVβ8 (F23.1, PharMingen) after the red blood cells were depleted with Red Blood Cell Lysis Buffer (Sigma, St. Louis, Missouri). FACS analyses were performed with a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences).

Animal Experimentation Procedures

All procedures described within were reviewed and approved by the Animal Experimental Committee at the RIKEN Institute and were performed in accordance with the RIKEN Guiding Principles for the Care and Use of Laboratory Animals.

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